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Molecular probing of wilt etiology in *Thuja orientalis*, and pathogen confirmation through Koch's Postulates

Muhammad Zulqar Nain Dara[1,2], M. Kaleem Sarwar[1,*] and Ahmad Nisar[1]

[1]Current Affiliation: College of Plant Protection, Jilin Agricultural University, Changchun 130118, China; [2]Department of Plant Pathology, University of Agriculture, University Road, Faisalabad, Pakistan
*Correspondent author's e-mail: kaleem.uaf@gmail.com

Thuja orientalis a monoecious widely grown ornamental plant in Pakistan. *T. orientalis* plants were observed being severely attacked by Fusarium wilt during a survey of the landscape areas of District Faisalabad. Infected plants had severe symptoms of leaves wilting, chlorosis, and necrosis. Wilt disease-affected samples of Thuja orientalis were collected from the University of Agriculture, Faisalabad, Pakistan, in 2017. The associated pathogen was identified based on morphological characters and molecular characterization by using ITS primers (ITS1-F/ITS4) specific to ITS regions of ribosomal RNA (rRNA) of the fungus. The morphologically characterized fungal isolates were used in pathogenicity. In the pathogenicity test, inoculated plants were compared with control plants (sprayed with distilled water). After 3-4 weeks, inoculated plants showed typical chlorosis symptoms. Pathogenicity test proved the *F. solani* as pathogen of wilt disease on Thuja.
Keywords: *Thuja orientalis*, Morpankh, *Fusarium solani*, Fusarium wilt, pathogenicity.

INTRODUCTION

Thuja orientalis (Common-name: Morpankh), also known as (*Platycladus orientalis, Biota Orientalis, Oriental arborvitae)* belongs to the family Cupressaceae. It is a coniferous evergreen plant that is monoecious and grown widely as an ornamental plant in Pakistan. It is distributed in Pakistan, China, Korea, Japan, India, Iran, and Russia. This plant is found in tropical and subtropical regions and is also an important medicinal plant with a substantial amount of borneol, bornyl acetate, thujone, camphor, and sesquiterpenes (Dan and Nhu, 1989). Biogenic oil is a significant natural product extracted from its leaves and used in fragrances, deodorizers, insectifuge, air fresheners, and aromatherapy (Lei *et al*., 2010). It also acts as an antioxidant, anticancer, and anti-inflammatory agent; alternatively, it can be used as insecticidal, molluscicidal, and nematicidal against different pests (Srivastava *et al*., 2012).

T. orientalis plants were observed being severely attacked by fusarium wilt during a survey at the University of Agriculture, Faisalabad, Pakistan, that were grown along roads as well as boundaries of grounds. Infected plant samples were collected from the University and the different nurseries of Faisalabad. Affected plants exhibited the symptoms of wilting, chlorosis, and stunting, and heavily infected plants were subjected to partial or complete death of the plant. Infected plant leaves gradually turned yellow and then subjected to apical necrosis. Premature defoliation was also observed in affected plants. Where the disease is advanced, infected plant leaves and branches become dried and dead gradually or in patches. Irregular and brown to dark-brown necrotic symptoms develop on leaves in an advanced stage of this disease. Then these necrotic lesions expand periodically and join together, making the whole leaf brown. Severely attacked plants occasionally become dead partially as well as completely.

MATERIALS AND METHODS

***Surface sterilization*:** Infected plant leaves samples exhibiting the symptoms of wilt collected from University of Agriculture Faisalabad, Pakistan and surroundings of Faisalabad city to isolate the associated pathogen with mild and severe and diseased plants of *Thuja orientalis*. Collected samples were brought into the FMB Lab (Fungal Molecular Biology Lab, University of Agriculture, Faisalabad, Pakistan). These samples were cut into small pieces (of ~5mm), rinsed with distilled water, followed by surface sterilization with 1% sodium hypochlorite solution for 1

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minute, and dried under axenic conditions. These sterile samples were then transferred to PDA (Potato Dextrose Agar) containing petri plates and kept at 25±2 ºC for 12 hours under light and dark conditions.

***Culturing for morphological identification*:** Fungal colonies that appeared from infected cuttings of affected leaves on PDA-containing petri plates were picked up gently by the single hyphal tip method and cultured separately. Isolated fungal colonies were cultured on PDA media and incubated at 25±2 ºC for a week to get a purified growth of these cultures. Mycelia, spores, and fungal masses of subsequently isolated fungal colonies were directly taken from purified cultures and studied on a morphological basis under the microscope. Cultures showed white to creamy growth on PDA medium with a thin layer of mycelial mat. Microscopic study showed that Microconidia was oval to reniform and ellipsoidal shape tapering at both ends. Macroconidia was wide, stout, and rough, as described by (Leslie and Summerell, 2006; Nelson *et al*., 1983).

Pathogenicity test: Pathogenicity was confirmed with the help of testing six months to 1-year-old young, healthy plants of T. orientalis following Koch's postulates. The conidial suspension was prepared, and 15 plants were inoculated by spraying, rubbing, and syringe inoculation methods. Then inoculated plants were covered with polythene bags and shifted to the greenhouse (at 25±2 ºC). After 24 hours, the polythene covering was removed, and inoculated plants were observed daily.

***Molecular Characterization*:** For molecular characterization, PCR analysis using ITS primers (ITS1-F/ITS4) (specific to ITS regions of ribosomal RNA (rRNA) of fungus) was performed. For DNA isolation, fresh fungal culture was established to get fresh mycelia. The fungal isolate was grown in potatoes dextrose broth at 28°C for 72 hours under a dark regime with shaking. Then mycelia were harvested by centrifugation and were subjected to DNA extraction using the protocol described by Plattner *et al*., (2009). A spectrophotometer quantified extracted DNA. PCR product was analyzed on 1% agarose gel through electrophoresis. Then PCR product was purified using a PCR purification kit and sequenced.

RESULTS

Wilt disease was observed on Thuja plants, and samples of wilt-infected leaves were collected (Fig.1a-1e). Then samples were transferred to PDA media containing plates to get fungal colonies. So, fungal colonies that appeared were purified and characterized morphologically (Fig. 2). Then morphologically characterized fungal pathogens were used in pathogenicity. In the pathogenicity test, inoculated plants were compared with control plants (sprayed with distilled water). After 3-4 weeks, inoculated plants showed typical chlorosis symptoms. They became withered and gradually subjected to death. At the same time, control plants did not show any symptoms and remained green until this experiment's end. *Fusarium solani* was re-isolated from symptomatic plants to fulfill Koch's postulates of pathogenicity. Various Fusarium species cause Fusarium wilt, and it was also reported on *T. orientalis* from India in 2007 (Raghavendra *et al*., 2007). So, molecular characterization was performed to validate morphological characterization. For this, extracted DNA was subjected to ITS-based PCR analysis and thereby amplicon of approx. 600 bp was achieved (Fig. 3). PCR product was purified and sequenced.

Figure 1a: Healthy plant of T. orientalis

Figure 1b: Development of wilt symptoms

Figure 1c: Symptoms of Fusarium wilt exhibited by *T. orientalis*

Figure 1d: Partially wilted plant

Figure 1e: Severely infected plant due to Fusarium wilt

Figure 2a: Growth pattern of isolated *Fusarium solani* from infected plants of *Thuja orientalis*

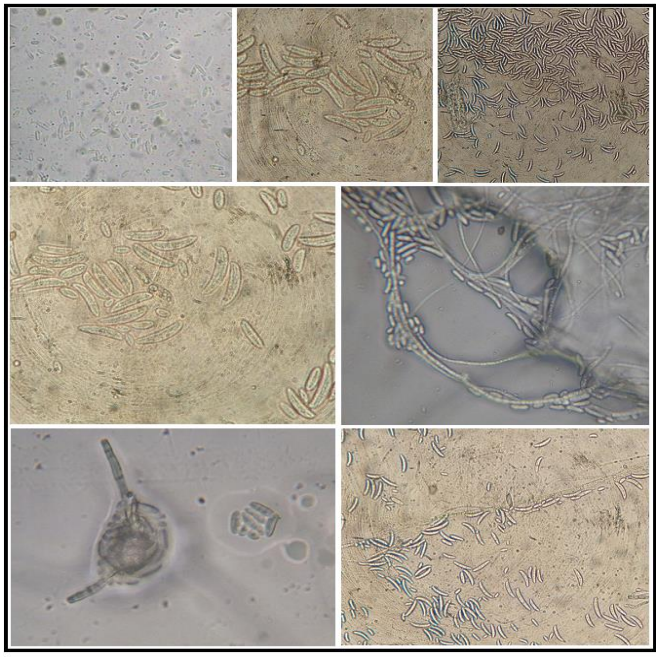

Figure 2b: Macro and microconidia of *Fusarium solani*

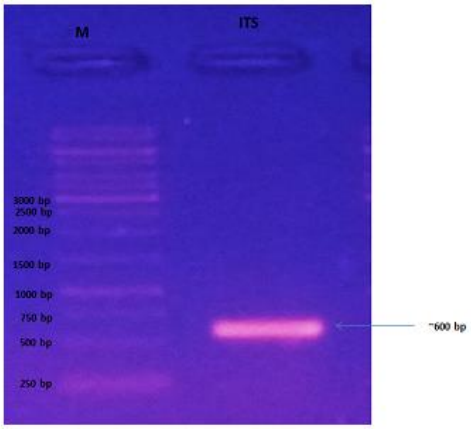

Figure 3: PCR amplification of ITS region

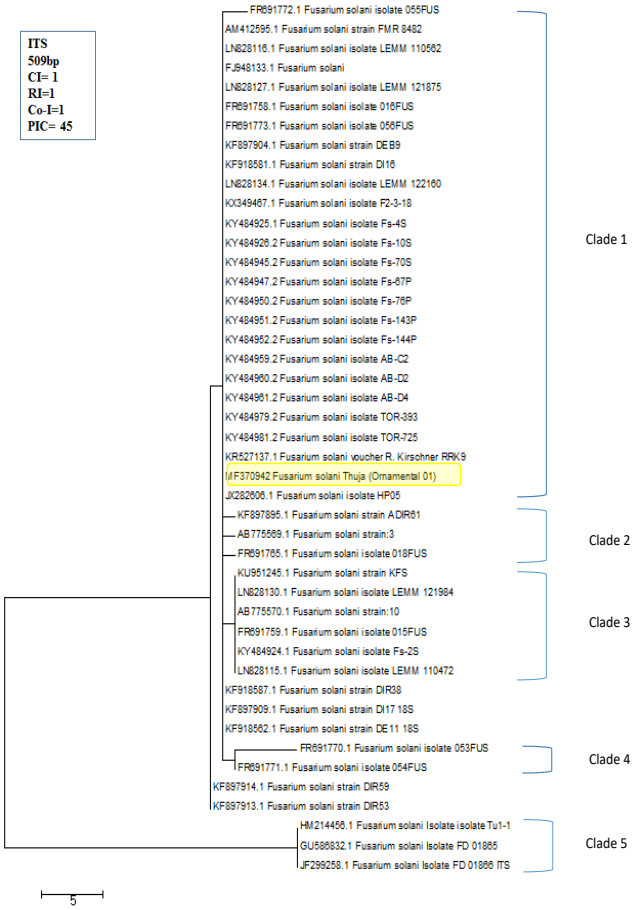

Figure 4. Maximum parsimonious tree: CI, Consistency index; RI, **Retention** index; Co-I, Composite index; PIC, parsimony-informative character.

Table 1. Percent Identity Matrix .

1: GU586832.1	100.00	99.46	100.00	93.57	94.35	93.96	93.96	93.96	94.15	93.96	93.96	93.96	93.96	93.96	93.96	93.94
2: HM214456.1	99.46	100.00	100.00	93.57	94.35	93.96	93.96	93.96	94.15	93.96	93.96	93.96	93.96	93.96	93.96	93.94
3: JF299258.1	100.00	100.00	100.00	93.45	94.24	93.85	93.85	93.85	94.04	93.85	93.85	93.85	93.85	93.85	93.85	93.85
4: FR691770.1	93.57	93.57	93.45	100.00	99.06	98.68	98.68	98.68	98.87	98.68	98.68	98.68	98.68	98.68	98.68	98.68
5: FR691771.1	94.35	94.35	94.24	99.06	100.00	99.63	99.63	99.63	99.81	99.63	99.63	99.63	99.63	99.63	99.63	99.62
6: FR691765.1	93.96	93.96	93.85	98.68	99.63	100.00	99.63	99.63	99.81	99.63	99.63	99.63	99.63	99.63	99.63	99.62
7: AB775569.1	93.96	93.96	93.85	98.68	99.63	99.63	100.00	99.63	99.81	99.63	99.63	99.63	99.63	99.63	99.63	99.62
8: KF897895.1	93.96	93.96	93.85	98.68	99.63	99.63	99.63	100.00	99.81	99.63	99.63	99.63	99.63	99.63	99.63	99.62
9: MF370942	94.15	94.15	94.04	98.87	99.81	99.81	99.81	99.81	100.00	99.81	99.81	99.81	99.81	99.81	99.81	99.81
10: KY484924.1	93.96	93.96	93.85	98.68	99.63	99.63	99.63	99.63	99.81	100.00	100.00	100.00	100.00	100.00	100.00	100.00
11: KU951245.1	93.96	93.96	93.85	98.68	99.63	99.63	99.63	99.63	99.81	100.00	100.00	100.00	100.00	100.00	100.00	100.00
12: LN828130.1	93.96	93.96	93.85	98.68	99.63	99.63	99.63	99.63	99.81	100.00	100.00	100.00	100.00	100.00	100.00	100.00
13: AB775570.1	93.96	93.96	93.85	98.68	99.63	99.63	99.63	99.63	99.81	100.00	100.00	100.00	100.00	100.00	100.00	100.00
14: FR691759.1	93.96	93.96	93.85	98.68	99.63	99.63	99.63	99.63	99.81	100.00	100.00	100.00	100.00	100.00	100.00	100.00
15: LN828115.1	93.94	93.94	93.85	98.68	99.62	99.62	99.62	99.62	99.81	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Based on homology, ITS sequences of known isolates of *Fusarium solani* from NCBI, and FUSARIUM-ID databases were retrieved and aligned with our GenBank deposited sequence (accession number MF370942). Sequence achieved was trimmed using Chromas software (version 2.6.4) and was then in silico characterized. For similarity, sequence was queried against GenBank and FUSARIUM-ID databases by

subjecting it to homology search tool BLAST (Basic Local Alignment Search Tool). The queried sequence got maximum similarities with subject sequences of Fusarium solani isolates and validated the result obtained through pathogenicity test. Therefore, this high quality, trimmed sequence was then submitted to GenBank with accession number MF370942. For this Multiple Sequence Alignment tool, Clustal Omega was used. Aligned sequences were then used for phylogenetic tree construction using MEGA6.06 software with maximum parsimony (Fig. 4).

Maximum parsimony analysis was performed to assess the relationship of queried sequence to ITS sequences of known *Fusarium solani* isolates and strains. Thereby single most parsimonious tree was achieved, which showed isolates nested in clade I have 100% similarity with our isolate (*Fusarium solani* Thuja (ornamental 01); accession number MF370942). While Fusarium isolates, those nested in remaining clades are phylogenetically diverse to our isolate. So, the percent identity matrix was generated by Clustal Omega software (Table 1) revealed that our Fusarium isolate (accession number MF370942) has 99.81% similarity with Fusarium isolates having accession numbers; FR691771.1, FR691765.1, AB775569.1, KF897895.1, KY484924.1, KU951245.1, LN828130.1, AB775570.1, FR691759.1, LN828115.1, 98.87% similarity with accession numbers FR691770.1; 94.15% similarity with accession numbers GU586832.1, HM214456.1 and 94.04% similarity with accession numbers JF299258.1.

Then phylogenetic tree with the neighbor-joining method was constructed, showing two sets comprising phylogenetically similar members. While remaining, all are independent and diverse from each other. Our queried entry (accession number MF370942) showed distinct and phylogenetically diverse status among them (Fig. 5).

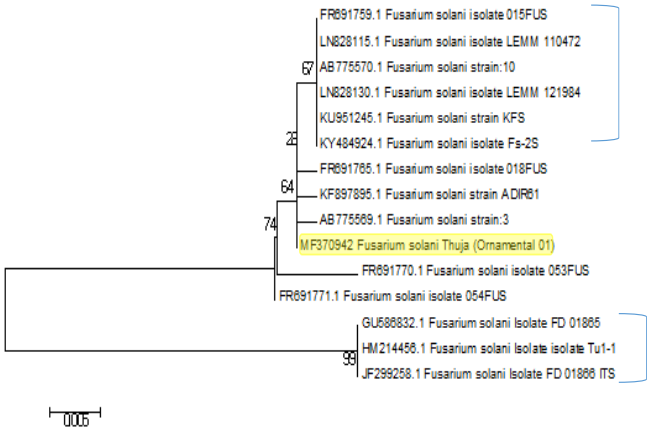

Figure 5. Phylogenetic tree constructed through neighbor-joining method

Based on the information created through the percent identity matrix, sequence alignment using BoxShade software was performed among the sequences, including the queried sequence and sequences (vouchered *Fusarium solani* isolates sequences from the FUSARIUM-ID database), which revealed the least similarity with our queried sequence (Fig. 6). This alignment revealed the variation based on nucleotide polymorphism that could be due to evolutionary forces such as mutation, deletion, base pair substitution etc. This showed the sequences, in comparison with our queried sequence, are of a more conserved sequence pattern. In contrast, the queried sequence has mutation and nucleotide variation at various sequence places, showing its distinctness and phylogenetic diversity. These results showed that Fusarium solani isolated from Thuja (ornamental plant) from the Faisalabad region is a different strain of this fungus which cause wilt disease particularly in Thuja.

```
MF370942      1 ------------------------------
GU586832.1    1 -----     A        T
HM214456.1    1 GGGTC     T        A
JF299258.1    1 ------------------------
consensus     1      ..... ........ ..........******************************

MF370942     31                                          C      CG  C GC
GU586832.1   56
HM214456.1   61
JF299258.1   37
consensus    61 ****************************************.******..**.*..**

MF370942     91  GC A     C               C   T      CTTCTG  TA
GU586832.1  116                                      --
HM214456.1  121                                      --
JF299258.1   97                                      --
consensus   121 **..*....*..***************.***.******.  .......************

MF370942    151
GU586832.1  174
HM214456.1  179
JF299258.1  155
consensus   181 ************************************************************

MF370942    211
GU586832.1  234
HM214456.1  239
JF299258.1  215
consensus   241 ************************************************************

MF370942    271
GU586832.1  294
HM214456.1  299
JF299258.1  275
consensus   301 ************************************************************

MF370942    331                        GC        C               C
GU586832.1  354                        -
HM214456.1  359                        -
JF299258.1  335                        -
consensus   361 ***********************.. .********.****************.*******

MF370942    391
GU586832.1  413
HM214456.1  418
JF299258.1  394
consensus   421 ************************************************************

MF370942    451          C
GU586832.1  473
HM214456.1  478
JF299258.1  454
consensus   481 *********.**************************************************

MF370942    511                         ----------
GU586832.1  533                           GCGGAGGA
HM214456.1  538                           --------
JF299258.1  514               ---------------------
consensus   541 **************............
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Figure 6. Multiple Sequence Alignment using BoxShade Software; Conserved sequence (*), conservative mutations (:), semi-conservative mutations (.), and non-conservative mutations ( )

Conclusions: Fusarium wilt on Thuja is wide spreading disease caused by the *F. solani* which was proved in this study. The fungus was characterized using morphological and molecular approaches and the characterized isolates proved as pathogen by completing the Koch's postulates. Further the bio control agents and fungicides further evaluated for the management of the disease.

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***Ethical statement*:** This article does not contain any studies with human participants or animal performed by any of the authors.

***Availability of data and material*:** We declare that the submitted manuscript is our work, which has not been published before and is not currently being considered for publication elsewhere.

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***Authors' contributions*:** Zulqarnain designed, completed the experiments and prepared the draft. M. kaleem and Nisar reviewed and finalized the draft.

***Consent to participate*:** All authors are participating in this research study.

***Consent for publication*:** All authors are giving the consent to publish this research article in PDC.

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